MATERIALS AND METHODS

1. Donors:

Voluntary healthy donors were tissue typed and blood from these donors was used for the study. The HLA phenotype of the responder [donor 1] cells was A2,3; B40; C6; DR2,4; DQ1,3; while that of the stimulator [donor 2] cells was A11,-; B37,51; Cw6; DR7,11; DQW2,7. The HLA phenotype of the 'third party' stimulator cells was A1,9; B27,51; C-,-; DR1,7; DQ2,3. Non-tissue typed individuals who responded to DR 5,7 were used on occasions.

2. Cell lines, fusion protein and monoclonal antibodies [mAb] :

T cell line :

Name	Source
H9 [T cell lymphoma]	ATCC HTB 176

B cell lines :

Name	DR haplotype	Source
SRBCL	DR 7,11	generated in our laboratory
SKBCL	DR 1, 7	generated in our laboratory
Daudi	DR 13b, Dw 19	ATCC CRL 7916
Herluf	DR 11,11	Prof. J. R. Lamb, London

Fusion protein : CTLA4-Ig, a recombinant chimeric fusion protein of the CTLA4 molecule and the constant region of human IgG [258], was used to stain for B7 family of ligands [gift of Dr Peter Lane, Basel Institute of Immunology, Switzerland].

Epstein-Barr virus [EBV] producing cell line : B95-8 cells (EBV transformed leukocytes of marmoset origin) [ATCC CRL 1612].

Monoclonal Antibodies :

Name	Source	Specificity	Isotype
W6/32	ATCC HB95	mouse anti-HLA-A,B,C.	IgG2a
L243	ATCC HB 55	mouse anti-HLA-DR	IgG2a
OKT 3	ATCC CRL 8001	mouse anti-human CD3 molecule	IgG2b
UCHT 4	Dr. Peter Beverley, London	mouse anti-human CD8 molecule	IgG2a
QS4120	Dr. Peter Beverley, London	mouse anti-human CD4 molecule	IgG
9.3	Dr.Alexander Rudensky, Seatle, USA	mouse anti-human CD28	IgG2b

3. Culture medium and other reagents :

3.1. Media :

Assays were carried out in RPMI-1640 (Gibco, USA) fortified with 2 mM L-glutamine (Sigma, USA) containing 5 x 10^{-5} M β-mercaptoethanol (Sigma, USA) streptomycin 100 μg/ml (Hi-Media, India) and 10% responder-autologous human serum (heat-inactivated at 56° C for 30 min). For maintenance of tumor cell lines, instead of human serum, 10% fetal calf serum (FCS) (Hyclone, USA) was used. CTLL-2, an IL-2 dependent murine T cell line, was maintained in DMEM (Gibco, USA), containing 10% FCS.

3.2. Phosphate-buffer saline (PBS) [259] :

NaH_2PO_4 1.9 mM

Na_2HPO_4 8.1 mM

NaCl 1.54 mM

pH adjusted to 7.2-7.4

4. Cryopreservation of cells :

Between 1-10 x 10^6 cells were frozen down in one vial. Cells were suspended at appropriate cell numbers in RPMI with 10% FCS, equal volume of freezing mixture [16% Dimethly sulfoxide (DMSO) in FCS] (DMSO from Sigma Chemical Co, USA) was added, mixed and aliquoted in 1 ml volumes in freezing vials. The freezing vials were

immediately transferred to -70°C freezer. After 48 h the freeze-downs were shifted to liquid N_2. For thawing frozen cells, the vials were quickly warmed in a 37°C water bath and cells transferred to 10 ml of RPMI, containing 10% FCS. After thorough mixing the cells were spun down at 100 x g, the supernatant discarded and after another similar wash the cells were used for experiments..

5. Cell preparation from peripheral blood :

5.1. Phlebotomy :

Peripheral blood was obtained from voluntary donors by venepuncture using a 21 gauge needle and syringes of appropriate volume. Heparin (Heparin, sodium salt, HiMedia Laboratories, India) was dissolved in PBS at a concentration of 200 I.U/ml, filtered for sterility and used as an anti-coagulant, at a final concentration of 5 I.U/1 ml of blood. Stocks solution of heparin was stored in a -20°C freezer.

5.2. Separation of peripheral blood mononuclear cells (PBMCs) from whole blood:

Peripheral blood was carefully layered on Ficoll-Paque density gradient medium (specific gravity 1.077, Ficoll-Paque, Pharmacia, Sweden). The ratio of blood to Ficoll was 1:1. The gradient was run at 400 x g for 20 min at 20°C., without brakes. The buffy-coat layer was carefully collected using a sterile pasture pipette into a centrifuge tube. The cells thus collected were diluted with RPMI with 10% FCS

(complete medium), by adding 2 volumes of the complete medium to one volume of the sample, mixed by inversion and centrifuged at 200 x g for 10 min at 20°C. The supernatant was then discarded and the pellet resuspended in about 10 ml of complete medium and given another wash. The supernatant was again discarded and the pellet resuspended in 5 ml of RPMI with 10% heat-inactivated autologous serum and the cells counted using a haemocytometer and trypan blue (0.4% solution in PBS, used 1:1 with cell suspension) as vital stain.

6. Cell fractionations :

[For fractionation protocols refer to flow-chart in Fig. 1]

6.1. Plastic adherence :

PBMCs were suspended in RPMI with 10% FCS at a concentration of 5 x 10^6 cells/ml. To sterile 100 mm tissue culture petri plates (Nunc, Denmark) 5 ml of the cell suspension was added. The plates were then incubated at 37°C in a 5% CO_2 incubator for 1 h with intermittent swirling to enable all cells to come in contact with the adhering surface. After incubation the non-adherent population was removed, the plate gently rinsed with RPMI without serum (plain RPMI) and the adherent population dislodged using gentle stream of complete medium, using a pipette.

6.2. Panning on antibody coated plates :

All pannings were done by coating 100 mm petri plates (Nunc,

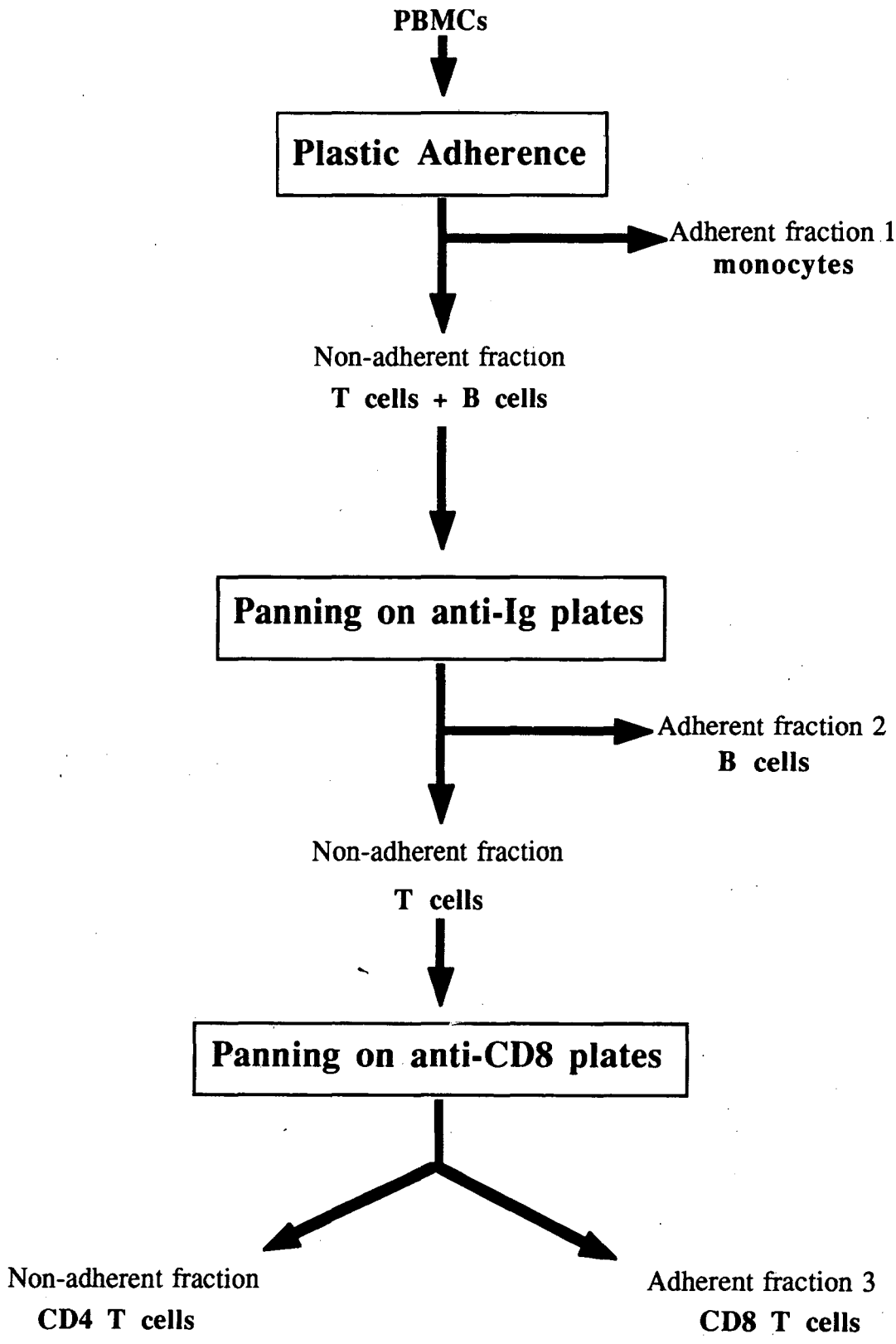

Figure 1. Flow-chart showing cell fractionation protocol: PBMCs were fractionated by plastic adherence, followed by panning on anti-Ig coated plates to obtain T cells. Adherent fraction 1 and 2 were pooled and called 'accessory' cell population. T cells were further panned on anti-CD8 coated plates to obtain CD4 T cells.

Denmark) with 10 μg/ml of the antibody in PBS for 1 h at 37° C, followed by washing with plain RPMI 1640 medium and incubation with 25 x 10^6 cells in complete RPMI per plate (5 ml in each case) at 37°C for 1 h. The plates were intermittently swirled to enable all cells to come in contact with the plate coated antibody. The non-adherent population was collected in each case, the plate rinsed with plain RPMI and the adherent population collected. To obtain T cells the plates were coated with affinity purified goat-anti-human Ig and the plate was incubated with the non-adherent population after plastic adherence exercise. The non-adherent population was practically devoid of HLA-DR positive cells and was termed as T cell population. The adherent population was pooled with the adherent population obtained after plastic-adherence and together they were called the 'accessory cell' population. To obtain CD4 T cells plates were coated with affinity purified anti-CD8 antibody (UCHT 4) and the plates incubated with purified T cells. The non-adherent population was collected as CD4 T cells, while the adherent population was termed as CD8 T cell population. Both the plastic adherence and pannings were repeated to obtain pure T, CD4 T or CD8 T cell population. The T cell populations were checked for the presence of accessory cell contaminants, by checking their responses to PHA. The purity of cell populations thus collected was assayed flow cytometrically after staining with anti-CD3 (OKT3), anti-CD4 (QS4120) and anti-CD8.

7. EBV transformation of B cells :

B95-8 cells (EBV secreting cell line) were cultured, culture supernatant (c/s) obtained and 1 ml aliquots were frozen down in liquid N_2. To 2 x 10^6 PBMCs 1 ml of EBV c/s was added and the volume made up to 5 ml with complete RPMI. The cells were then incubated at 37°C in a 5% CO_2 atmosphere for 2 h after which the 5 ml of medium was added and the cells plated in a 6 well plate (Nunc, Denmark) at 2 ml / well. The plates were left in the incubator for about three weeks, by which time the transformed B cell clusters started appearing. These were then expanded in RPMI with 10% FCS, tested for expression of MHC class II, and freeze-downs were made.

8. Making of hybridoma culture supernatants :

Hybridoma cells were cultured in RPMI with 10% FCS, and cultures expanded. The expanded cultures were then allowed to overgrow, till the medium turned yellow. The cells were then separated by centrifugation and the amount of mouse Ig in the culture supernatant estimated. The c/s were then stored as aliquots in a -20°C freezer or the mAbs purified on an affinity column.

9. Assay for mouse Ig in hybridoma culture supernatant :

Mouse Ig was assayed in hybridoma culture supernatant by EIA (Enzyme Immunoassay). 10 μg per well of affinity purified goat anti-mouse Ig in carbonate-bicarbonate buffer, pH 9.6, was coated onto 96

well ELISA plates (Becton & Dickenson, USA), by incubating overnight at 4°C. The plates were then washed 3 times with washing buffer (PBS containing 0.5% of Tween-20) [Merck, India], and blocked with washing buffer containing 1% Lactogen (Nestle, India). After another round of washing 100 µl of serial dilution of c/s was added and incubated for 1 h at 37°C. The plates were again washed and horse raddish peroxidase (HRP)-conjugated goat anti-mouse Ig reagent, at the appropriate dilution, was added to the well, and the plates were again incubated at 37°C for 1 h. After washing 100 µl/well of substrate solution [0.15 M citrate phosphate buffer, pH 5.0 containing 0.5 mg/ml o-phenylene diamine (Sigma Chemical Co.,USA) and 1µl/ml of hydrogen peroxide (Qualigens, India)] was added and the color was allowed to develop in the dark. Further color development was arrested by adding 50 ml of 5 N sulfuric acid (Qualigens, India) and absorbance measured at 490 nM in an ELISA reader (Bioteck, USA). To obtain a standard curve, known quantities of mouse Ig were run in parallel in the assay and a standard curve was plotted. Concentration of Ig was calculated in terms of µg/ml of mouse Ig.

10. Affinity purification of mAb from hybridoma c/s :

The mAb culture supernatant was spun at 20,000 x g and 4°C and filtered through a 0.45 µm filter. After dialyzing against phosphate buffered saline (PBS), pH 8.0 solution, the c/s was passed through a protein A-Sepharose column (Bio-Rad, USA), till no antibody was detected in the eluate (checked for by ELISA). The column was then

washed with several volumes of PBS, pH 8.0, after which the bound antibody was eluted with 0.1 M citric acid (Qualigens, India) at an appropriate pH, using 0.1 M NaOH (Qualigens, India). For mouse IgG1 and IgG2a, pH 6.5 and pH 4.5 respectively were used, and for IgG2b and IgG3 , pH 3.0 was used. The eluate was then pooled and dialyzed against PBS, pH 7.3, quantitated, aliquoted appropriately, checked for the desired specificity and stored at -20°C.

11. Activation of T cells:

11.1. Activation of *ex vivo* human T cells :

T cells were activated with PHA in the presence of irradiated accessory cells. Purified T cells were mixed with γ-irradiated (2000 rads from a ^{60}Co source) accessory cells at a ratio of 10:1 (T cells : accessory cells) and the cell pellet was resuspended in RPMI containing 10% autologous serum, to obtain 1 x 10^6 cells/ml. This cell suspension was added to a 6-well tissue culture plate (Nunc, Denmark), so as to get 5 x 10^6 cells/well. Recombinant IL-2 (rIL-2) (Boehringher Mannheim, Germany) was added at a final concentration of 50 units/ml. Occasionally T cells were activated with plate coated anti-CD3 mAb. For this, to each well in a 6 well plate (Nunc, Denmark) 1 ml of affinity purified anti-CD3 (OKT 3) at 10 μg/ml in PBS was added. The plates were incubated in a 5% CO_2 incubator at 37°C for 1 h, the antibody solution removed and the unbound antibody removed by rinsing gently with plain RPMI. The cell suspension was now added to these wells and

rIL-2 at a final concentration of 20 U/ml was incorporated. The cells were activated by either protocols for 48 h to obtain optimum expression of MHC class II on T cells. To obtain long term T cell lines, purified T cells were activated with PHA and IL-2 maintained in culture for a week after which they were harvested, washed and treated with PHA and IL-2 as the case may be, along with addition of fresh γ-irradiated syngenic accessory cells.

11.2. Activation of human tumor T cell line H9 :

H9 cells were activated *in vitro* with PHA (0.3 μg/ml) and PMA (Phorbol Myristate Acetate) [Boehringher Mannheim, Germany] (10 ng/ml). The expression of MHC class II was monitored by flowcytometry and optimum expression of MHC,class II was obtained at 60 h.

12. Flow cytometric analysis :

12.1. Staining for flowcytometry :

Staining was done on ice by incubation of 1 x 10^6 cells with 100 μl of culture supernatants of appropriate mAb, in 96 well round bottom polystyrene plates (Tarsons, India), for 1 h . Control samples were incubated with washing buffer (1x PBS containing 1% BSA and 0.1% sodium azide (Merck, India)) or with an appropriate isotype matched control antibody. The cells were washed thrice with washing buffer, chilled to 4°C before use. After this the cells were incubated with

either goat anti-mouse Ig-fluorescein or goat anti-human IgG-fluorescein, depending upon the first antibody used. For amplification of low-level staining, a rabbit anti-goat Ig antibody was added after the above protocol, followed by goat anti-rabbit Ig-fluorescein. After washing cells were analyzed on a flowcytometer (EPICS 751, Coulter, USA). Data were collected from 10,000 cells in each sample. When the analysis could be not done immediately the stained cells were fixed using 0.1% paraformaldehyde (Merck, India) in 1x PBS and analyzed later after washing off the paraformaldehyde.

12.2. Conjugates :

Goat anti-mouse Ig-FITC conjugate (NII, New Delhi)

Goat anti-human Ig-FITC conjugate (NII, New Delhi)

Goat anti-human IgG-FITC conjugate (Southern Biotechnology Associates Inc., USA)

Mouse anti-human MHC class II conjugated to FITC (L234) (Becton & Dickinson, USA)

Mouse anti-human CD3 conjugated to FITC (OKT 3) (Becton Dickenson, USA)

FITC conjugated isotype controls (Becton & Dickinson, USA)

13. Paraformaldehyde fixation of APCs :

A stock solution of 1% paraformaldehyde (tissue culture grade from Sigma, USA) was made in PBS and filtered sterile and stored at 4°C. PBMCs were washed with plain RPMI to remove FCS, and

resuspended in minimal volume of plain RPMI (about 500 µl for 10^6 cells) and appropriate volume of 1% paraformaldehyde stock solution was added to obtain the desired final concentration of 0.01%. The cells were then incubated for 20 min at room temperature (R.T), with intermittent shaking. After incubation the cells were pelleted by centrifugation and the paraformaldehyde solution removed. About 10 ml of complete medium was for washing the cells. Two more washes were given and the cells were resuspended in RPMI containing autologous (inactivated) serum and used.

14. T cell proliferation assays :

14.1. T cell proliferation assays :

T cell responses were assayed by proliferation assays using 96-well plates (Nunc, Denmark) in 200 µl cultures. PBMCs, purified T cells or CD4 T cells were used as responder cells at 1 x 10^5 cells per well after initial titrations for optimizations. Stimulator cells were used after γ-irradiation (2000 rads for non-transformed cells, 12000 rads for tumor cells, from a ^{60}Co source) at various concentrations as shown. After doing a time course the optimum time period for primary proliferation assays and restimulation assays were determined. The plates were incubated in 5% Co_2 incubator at 37°C, for 4 days for restimulation assays and 6 days for primary proliferation assays using non-transformed cells as stimulators and 48 h for primary proliferation's using tumor cells as stimulators, and pulsed with 0.5 µCi ^{3}H-thymidine

(^{3}H-TdR, Amersham, UK) per well during the last 12-16 h of the assay. ^{3}H-TdR incorporation was assayed after harvesting the cultures onto glass-fiber filters for scintillation spectroscopy (Betaplate, LKB-Pharmacia, Sweden). For proliferation assays in the presence of anti-CD28 mAb, affinity purified antibody was added to the priming cultures at a final concentration of 1 μg/ml of the antibody. The results are shown as mean counts per minute (cpm) of triplicate cultures along with standard errors of mean. In experiments where secretion of IL-2 in primary proliferation's were done, 50 μl of c/s was collected in to a 96 well tissue culture plate.

14.2. Priming *in vitro* :

For priming *in vitro*, 10 x 10^6 responder PBMCs, purified T cells or CD4 T cells were incubated for 72 h at a ratio of 1:1 with various allo-APC populations, (γ-irradiated PBMCs or activated T cells) in 3 ml cultures in 6-well plates (Nunc, Denmark). The time duration for the priming and the ratio of stimulator cells added was determined after extensive titrations. At the end of this period, cells were harvested, washed extensively, viable cells were counted and used as responders in a restimulation assay with γ-irradiated PBMCs or activated T cells as APCs. For priming in the presence of anti-CD28 mAb, affinity purified antibody was added to the priming cultures at a final concentration of 1 μg/ml of the antibody.

14.3. Bioassay for IL-2 :

CTLL-2, an IL-2 dependent mouse T cell line was used to measure IL-2 levels in the c/s harvested from primary T cell alloresponse assays. CTLL-2 cells were grown in presence of 2 U/ml of rIL-2, in complete medium (DMEM with 10% FCS). The cells were washed and resuspended in complete medium without IL-2 for 6 h before the assay. To 50 µl c/s collected from the T cell proliferation assay, CTLL-2 cells were added at 1 x 10^4 cells/well. A titration of rIL-2 done in parallel served as a positive control for quantitation of the amount of IL-2 present in the c/s. The plates were pulsed with 0.5 µCi ^{3}H-thymidine per well during the last 12 h of the 40 h assay, harvested on glass-fiber filters for counting by scintillating spectroscopy.

15. RNA extraction and dot -hybridization :

15.1. Reagents :

The following precautions were observed while working with RNA,

1] All glassware was baked at 300^{o}C for 4 h.

2] All plastic ware such as pipetman-tips, eppendorf tubes were soaked in DEPC (USB, USA) water [0.1% DEPC (Diethyl pyrocarbonate) in distilled H_2O] (USB, USA) overnight and autoclaved.

3] All solutions were made in autoclaved DEPC water and then autoclaved. Only ultra-pure reagents that specially reserved for RNA work only were used.

4] At all times disposable gloves were worn while working with RNA to prevent contamination with RNAases.

Composition of reagents :

a] Denaturing solution : 4 M guanidine thiocyanate (USB, USA), 25 mM sodium citrate (BDH, India) (pH 7.0), 0.5% sarcosyl (Sigma, USA)and 0.1 M β-mercaptoethanol [USB, USA).

b] 2 M NaOAc [BDH, India], pH 4.0

c] Chloroform : isoamyl alcohol (Qualigens, India), [49:1]

d] Water-saturated ultra-pure phenol (Qualigens, India), stored at 4°C

e] 100% isopropanol (Qualigens, India)

f] 75% ethanol (Qualigens, India)

g] TE (Tris-EDTA) [10 mM Tris-Cl (Pharmacia, Sweden), 0.1 mM EDTA [ethylenediaminetetraacetic acid] (Qualigens, India), pH 7.4]

h] 20x SSC : NaCl (Qualigenes, India), 3 M, Na citrate (Qualigens, India) .3M, pH 7.0

i] 5 x Denhart's solution : 1% Ficoll (Type 400, Pharmacia, Sweden), 1% Polyvinylpyrrolidone (Sigma, USA) and 1% BSA (Fraction IV, Sigma, USA).

15.2. RNA extraction :

Cultured cells were harvested, spun down in a centrifuge tube and then washed with plain RPMI. To the pellet of about 10^7 cells 1 ml of denaturing solution was added and the cells were passed through a pipette seven to ten times to lyse them. The homogenate was then

transferred to two eppendorf tubes (500 µl/tube) and 50 µl of 2 M sodium acetate, pH 4.0 was added and mixed thoroughly by inversion. Then 500 µl of water-saturated phenol and 50 µl of chloroform:isoamylalcohol were added, mixed thoroughly by vortexing and incubated for 15 min on ice. The tubes were then spun for 20 min at 10,000 x g, at 4°C. The aqueous phase was transferred to a fresh tube and precipitated by addition of 1 volume of 100% isopropanol. The samples were placed for 2 h at -20°C. After this the tubes were centrifuged at 10,000 x g at 4°C and the supernatant was discarded. The precipitate was dissolved in 300 µl of denaturing solution and re-precipitated with 1 volume of isopropanol. The precipitate was then washed with 75% ethanol and dissolved in 100 µl of TE. The RNA was then quantitated by measuring its absorbance at 260 nm and 280 nm. An A_{260}/A_{280} ratio of 1.8-2.0 indicated good purity of RNA.

15.3. RNA dot blotting :

a] Set up membrane for transfer :

The slot-blot manifold (Bio-Rad, USA) was cleaned with 0.1 M NaOH and rinsed with distilled water (DEPC treated). A piece of nitrocellulose membrane (Bio-Rad, USA) was cut to the size of the manifold and placed in a dish containing 20x SSC and allowed to submerge. The membrane was allowed to soak for 30 min and placed in the manifold on top of two sheets of Whatman 3 MM (Whatman, USA). The manifold was then assembled, the slots filled with 10x SSC and checked for leaks.

b] Denaturing of RNA samples :

To 1 volume of RNA samples 3 volumes of solution (50% formamide, 7% formaldehyde and 1x SSC in DEPC water) was added and incubated at 65°C for 15 min and chilled on ice. Two volumes of ice-cold 10x SSC was added to each sample. Doubling dilution of each sample was done in ice cold 15x SSC using a sterile 96 well round-bottom plate (Nunc, Denmark).

c] Slot-blotting :

Suction was now applied to the manifold and the 10x SSC added in step (a) was allowed to filter through. Samples were the applied to the appropriate slots and allowed to filter through. To each well 1 ml of 10x SSC was added and allowed to filter through. This was repeated once more. The apparatus was dismantled and the membrane was placed on a sheet of Whatman 3 MM paper and allowed to dry.

d] Immobilization of the RNA :

The dried membrane wrapped in aluminum foil were baked in a vacuum oven at 80°C for 2 h before hybridization.

15.4. Oligonucleotides probes used and their labeling :

1] Oligonucleotide probe for human Ii mRNA : Hi-22 (22-mer)

5' AGATAAGGTCGCGCTGGTTCATC 3'

2] Oligonucleotide probe for human β-actin mRNA : 29-mer

5' CTAGAAGCATTGCGGTGGACGATGGAGGG 3'

3] 5' End-labeling of oligonucleotide with T4 Polynucleotide Kinase :

A reaction mixture was set up [oligonucleotide (100 ng approximately) 3 µl, γ-^{32}P ATP 3000 Ci/mol at 10 µCi/ml 3 ml, forward exchange buffer 5 µl (New England Biolabs, USA) and T4 kinase enzyme (New England Biolabs, USA) 8-10 u/ml 1 µl and distilled water (D/W) 8 µl] and incubated at 37°C for 1 h. The reaction was stopped by addition of 5 µl of 0.5 M EDTA.

4] Purification of labeled oligonucleotide from the mixture :

A 0.2 ml DE-52 (Pharmacia, Sweden) column was made and washed with 2 ml of TE. The kinased oligonucleotide mixture was loaded on the column, washed with 2 ml of TE, followed by another wash with 2 ml of 0.2 M NaCl in TE and eluted in 1 ml 1 M NaCl in TE.

15.5. Hybridization and auto radiography :

The membrane was pre-hybridized in pre-hybridization solution (50% formamide, 6x SSC, 5 x Denhart's and 50 µg/ml of salmon sperm DNA) at 65°C for 4h. Labeled probe was added to this mixture and hybridized overnight at 65°C. After hybridization the membrane was washed twice with 6x SSC for 15 min at R.T. and setup for autoradiography (using Du Pont X-ray cassettes with Cronex

intensifying screens, USA and Kodak X-ray Films, Kodak Eastman Company, USA). The autoradiographs were developed at different time points to obtain clear images (developer and fixer obtained from Agfa, India).